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<p>(54) Title: DNA FOR DIAGNOSING PNEUMOCYSTIS CARINII</p>		
<p>5' - G A T G G C T G T T T C C A A G C C C A - 3' 5' - G T G T A C G T T G C A A A G T A C T C - 3'</p> <p style="text-align: right;">(I)</p>		
<p>(57) Abstract</p> <p>A method of assaying a sample of DNA from respiratory secretion of a patient for <i>Pneumocystis carinii</i>, comprises amplifying a polynucleotide sequence derived from <i>P. carinii</i> by a polymerase chain reaction, and detecting the amplified sequence present. Two DNA sequences are given, together with a number of pairs of oligonucleotide primers, including particularly the pair (I).</p>		

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DNA FOR DIAGNOSING PNEUMOCYSTIS CARINIIIntroduction

5 Pneumocystis carinii is established as the
prime cause of opportunistic pneumonia in patients with
AIDS and those immunosuppressed on oncology and
transplant units. Debate over the taxonomy of
10 P. carinii continues and the fastidious nature of the
parasite still demands the use of microscopy after
histochemical staining or immunofluorescence for the
detection of the parasite in diverse forms of lung
samplings [1,2]. The requirement for observer
15 expertise is significant and limits the diagnostic
power of these techniques. There are sound theoretical
grounds for believing that DNA amplification using the
polymerase chain reaction (PCR) [3] might provide both
a specific and sensitive means of identifying the
20 parasite in clinical samplings and one that finally
might be amenable to automation. Other applications of
DNA amplification to the study of the epidemiology of
P. carinii infection are also evident.

European Patent Specification 327390
describes DNA sequences produced by recombinant DNA
25 technology from an experimental rat model which
hybridise to DNA of P. carinii but not to mammalian DNA.
These DNA sequences were present as inserts in plasmids
of which two were designated pAZ 102 and pAZ 112. This
invention results from further work described herein:-
30 A. We have sequenced the inserts of pAZ 102 and
pAZ 112. The sequences are set out in Figures 1 and 3.
B. Using suitable oligonucleotide primers we
have amplified, by the polymerase chain reaction (PCR)
technique, P. carinii DNA from infected rat lung
35 samplings, to an extent that the amplified DNA was

easily detectable by staining an electrophoresis gel. In the same way, we have amplified P. carinii DNA from infected human lung samplings.

5 C. Analysis of the amplified DNA from human samples has shown significant sequence differences from the infected rat material. The human sequence is set out in Figure 1, and the similarities and differences between human and rat sequences are highlighted.

10 D. The human sequence, leads to improved oligonucleotide primers which more efficiently amplify P. carinii DNA of human origin.

15 E. We have developed a more sensitive detection system, involving hybridising the amplified DNA to a labelled probe which probe is part of the sequence determined in C. intermediate the two primers. By these means we are able to detect P. carinii DNA, and thus to diagnose infection by P. carinii, in patients who do not (yet) show clinical symptoms.

20 In one aspect, this invention provides, as new chemical compounds, the nucleic acid sequences shown in Figures 1 and 3, single and double chain fragments thereof at least 15 nucleotides in length, and nucleic acid sequences and fragments having at least 90% homology thereto. These result from steps A. and C. above.

25 In another aspect, the invention provides a method of assaying a sample of DNA from respiratory secretion of a patient possibly infected with Pneumocystis carinii, which method comprises using a pair of oligonucleotide primers based on the sequences shown in Figure 1 or Figure 3 to amplify by a polymerase chain reaction a polynucleotide sequence derived from P. carinii if such sequence is present in the sample, and detecting the amplified sequence if present. This is step B. above, and steps D. and E. 35 constitute preferred features of the method.

For maximum efficiency and specificity of the PCR reaction, the choice of oligonucleotide primers is critical. The primers must be based on the sequence to be amplified and may be identical to the two ends.

5 However, identity is by no means essential (R. Sommer and D. Tautz, *Nucleic Acids Research*, Vol. 17, No. 16, 1989, p. 6749). Generally the two or three nucleotides at the 3'-end of the primer, and at least 50% (preferably at least 90%) of all the nucleotides of the primer, are homologous to the sequence to be amplified.

10 The primers are partly or completely homologous to particular sites of the sequence to be amplified. For maximum efficiency of the PCR reaction, the location of those sites is also important. Although most primers will work with varying degrees of
15 success, general guidelines for obtaining useful primers are found in the literature (see Saiki R. K. et al., *The Polymerase Chain reaction in Genome Analysis* (Ed K. E. Davies) IRL, Oxford, 1988). However, the design of effective primers tends to be empirical.
20 Described below are one pair of primers derived from pAZ 102 that have proved outstanding; and several pairs of primers derived from pAZ 112 that have proved effective.

In other respects, the PCR conditions may be
25 conventional. The primers may be at least 8, conveniently about 20, nucleotides in length. The number of cycles required to achieve sufficient amplification may be from 15 to 50. If required to improve specificity, two different pairs of primers may
30 be used. The resulting amplified sequence has a predetermined length, and moves a predetermined distance on an electrophoresis gel. The resulting band can be visualised, either by conventional staining techniques, or by hybridisation to a labelled probe
35 which probe is homologous to part or all of the known

sequence being amplified.

Reference is directed to the accompanying Figures, in which:-

Figure 1 comprises sequence data on different DNA samples. Row 1 entitled "Rat" is from lung samplings from a rat infected with *P. carinii*. Row 2 entitled "Human" is from lung samplings of infected humans. Secondary structure has been taken into consideration and gaps (-) introduced to obtain maximum alignment. Numerous differences between human and rat sequences are shown boxed.

Figure 2 is a diagram of the circular plasmid pAZ 112 showing certain features including the positions of polynucleotide primers used in PCR.

Figure 3 comprises the complete sequence of the insert of pAZ 112, with the oligonucleotide primers marked. R/C means reversed and complemented, i.e. the actual sequence of the primer is the reverse and complementary to that marked.

Table 2 lists the oligonucleotide primers referred to in Figures 2 and 3.

Table 3 lists the primer combinations successfully used by us and the approximate size of the resulting amplification product.

The following Examples illustrate the invention. Example 1 relates to DNA from the plasmid pAZ 102 whose sequence is shown in Figure 1. Example 2 relates to DNA from the plasmid pAZ 112 whose sequence is shown in Figure 3. Example 3 reports a clinical trial following the method of Example 1.

Example 1

Methods

Cloning and sequencing of part of the gene coding for the large sub-unit of the mitochondrial ribosomal RNA from *P. carinii*

P. carinii pneumonia was induced in the rat model and DNA extracted and cloned from a parasite enriched fraction as previously described [4]. *P. carinii* specific sequences were confirmed by characteristic in situ hybridisation patterns and recombinant plasmid pAZ102 was selected as a candidate mitochondrial sequence because of strong signals derived in dot blot hybridisation studies on infected samples. The recombinant plasmid pAZ102 (insert 570 bp) was sequenced using Sanger's chain termination method and the Sequenase kit (United States Biochemical Corporation, Cleveland, USA), ³⁵S (Amersham, UK), Sequagel (National Diagnostics, Marville, USA). The DNA sequence was compared with those available in several databases including EMBL and Genbank. From the sequence data on pAZ102 and comparative analysis of the databases, the fragment was identified as a portion of the gene coding for the large sub-unit of the mitochondrial, ribosomal RNA of *P. carinii* and this showed significant homology with fungal sequences (manuscript in preparation).

Oligonucleotide primers

Two sequences of moderate conservation that were specific to *P. carinii* were selected for construction of oligonucleotide primers for the polymerase chain reaction:- pAZ102 - E:-5'-GATGGCTGTTTCCAAGGCCA-3'; pAZ102-H:-5'-GTGTAGTTGCAAAGTACTC-3'. An oligonucleotide for confirmatory Southern hybridisation on amplification products was chosen, pAZ102-L1. Subsequently a new internal oligonucleotide specific to human *P. carinii* sequences was constructed, pAZ102-L2 (Table 1).

Template DNA

- i) Samplings for DNA amplification using our oligonucleotide primers comprised a) pulmonary lavage samplings from 3 humans and 3 rats with *P. carinii* pneumonia documented by methenamine silver staining and microscopy, and b) isolates from a series of organisms including some

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potential pulmonary pathogens: Candida (an albicans and a non-albicans strain), Cryptococcus neoformans, Mycobacterium tuberculosis, Saccharomyces cerevisiae and Aspergillus nidulans.

- ii) Template DNA was prepared from each sample by proteinase K digestion in the presence of SDS and EDTA followed by phenol/chloroform/ether extraction, and ethanol precipitation.

DNA amplification

Using primers pAZ102-E and pAZ102-H the template samples, together with control samples without template underwent 40 cycles of amplification performed with denaturation at 94°C for 90 seconds, annealing at 50°C for 90 seconds and extension at 72°C for two minutes (Techne, UK). The DNA amplification reaction mixture (50 µl) contained 50mM KCl, 10 mM Tris, pH 8, 0.01% (w/v) gelatin, 3mM MgCl₂, 400 µM dNTPs (Boehringer Mannheim, UK), 0.4-1.0 µM oligonucleotide primer and 3 units of Amplitaq (Perkin Elmer Cetus, UK).

To avoid the possibility of false negative results in the human clinical samples, i.e. failure to detect the specific amplification product for technical reasons, we carried out a parallel polymerase chain reaction on each sample using primers derived from the human anti-thrombin gene, exon 2

These primers, (AT1: -5'-

GTTGCAGCCTAGCTTAAGTTGGCA-3'; AT4: -5'-GGTTGAGGAATCATTGGACTTG-3') allowed amplification to take place using human genomic DNA as template. In each of the clinical samples the 500 bp specific product was detected, demonstrating efficient amplification.

The potential problem of contamination in PCR was monitored by systematic use of the following techniques: i) including several negative control samples with no added template DNA; ii) by the use of UV-irradiation of the PCR reaction mixes prior to the addition of the template DNA [5]; iii) the use of separate disposable microcapillaries for the addition of each template (Laser Laboratory Systems Ltd, UK). The control

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samples remained negative in all experiments.

Amplified products (10 μ l) were electrophoresed in 1.5% agarose gels and visualised after ethidium bromide staining by ultraviolet light. The gel was Southern blotted on to Hybond N (Amersham, UK) and hybridised with 32 p end-labelled internal primer at 46°C (pAZ102-L1) or 40°C (pAZ102-L2) for 3 hours[6]. Filters were subsequently washed at high stringency at 54°C (pAZ102-L1) or 48°C (pAZ102-L2) and filters exposed to radiographic film at -80°C with intensifying screens. The expected amplification product was 355bp long in the rat derived parasite, that from the human derived parasite being 9 bp shorter.

Sequencing of products of DNA amplification

The PCR product was gel purified and recovered from the agarose gel using GeneClean (Bio 101, Inc). The purified DNA was heat denatured and sequenced as described above using primers pAZ102-H or pAZ102-E at 20pmole/ μ l.

Results

The oligonucleotide primers derived from rat P.carinii produced efficient amplification of specific sequence from both rat and human hosts, shown by ethidium bromide staining but none from the range of other organisms including some potential pulmonary pathogens.

The internal oligonucleotide, pAZ102-L1, derived from the rat P.carinii, produced strong hybridisation signals on Southern hybridisation with amplified products from the infected rat lungs, but weak signal, at high stringency, with the amplified product derived from human samples although these were visible on ethidium bromide staining. Direct sequencing of the amplified products from each of the rat and human samples allowed comparison of their sequence and demonstrated limited but consistent differences between the P.carinii DNA from these two hosts which included 5 base changes in the sequence of the internal oligonucleotide pAZ102-L1 (Table 1).

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An oligonucleotide specific to the human derived organisms was constructed, pAZI02-L2, which showed strong hybridisation with the amplified product from human P.carinii and conversely showed weak hybridisation with the rat P.carinii amplified product. It produced no hybridisation with the PCR products of the range of other organisms tested.

Studies using serial dilutions of human derived P.carinii template DNA indicated that the application of oligoblotting with pAZI02-L2 to amplified DNA products increased the sensitivity of detection by at least 100 fold over visualisation by ethidium bromide staining.

The P.carinii oligonucleotide primers successfully amplified specific PCR product from bronchoscopic alveolar lavage samplings from 10 HIV-positive individuals with pneumocystis pneumonia as documented by positive methenamine silver staining on the lavage samples. Lavage samples from 5 immunocompetent subjects were studied as controls. These failed to show specific PCR product by ethidium bromide staining or oligoblotting.

Discussion

We have characterised a portion of the gene coding for the large sub-unit of mitochondrial, ribosomal RNA from P.carinii and comparative analysis of this indicates significant homology with fungal sequences. This result accords with data we have on other mitochondrial genes and the observations of other groups on ribosomal RNA[7].

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We have identified P.carinii specific sequences from which we have constructed oligonucleotide primers which allow efficient amplification of part of this ribosomal RNA gene from P.carinii infecting both rat and human hosts but not from a range of other organisms including some potential pulmonary pathogens. These results indicate the specificity of the amplified products to P.carinii, confirmed on Southern hybridisation with internal oligonucleotide pAZ102-L1 derived from the rat sequence and applied to the rat pulmonary samplings. Results from the human samplings suggested the likelihood of differences in sequence between the amplified products from the rat and human. This was confirmed by comparison of sequences which indicated limited, but consistent differences.

This finding is highly relevant to the hopes for developing DNA amplification as a diagnostic tool in clinical medicine. We have shown that our oligonucleotide primers can be used to identify the presence of P.carinii in a number of bronchoalveolar lavage samples. By using our second internal oligonucleotide, pAZ102-L2, which is specific to human P.carinii, the sensitivity of detection of amplified product is considerably increased. This method shows great potential for use on non-invasive samples such as induced sputum where parasite numbers are lower. It will not only be valuable in diagnosis but also in addressing questions relevant to the epidemiology of P.carinii.

The application of DNA amplification to diagnosis will require careful calibration to ensure that levels of P.carinii in keeping with clinical pneumonia can be distinguished from lesser degrees of colonisation that are likely to occur in the immunodeficient before clinical disease is manifest. Such methods of calibration of DNA amplification are becoming available [8,9] and their application to diagnostic studies on P.carinii in diverse clinical samplings, including lavage and induced sputum, are now required.

Example 2

For pAZ 112 we used the techniques described in Example 1, and the oligonucleotide primers given in Table 2 in the combination set out in Table 3. We
5 achieved amplification by PCR of the sequences of pAZ 112 shown in Figure 3.

Example 3

10 Clinical Specimens

Alveolar lavage samples were obtained from 47 patients investigated by bronchoscopy at the Churchill
Hospital, Oxford, and at the Middlesex Hospital,
15 London.

Thirty seven patients were immunosuppressed, either by HIV infection (33) or by treatment for lymphoma (2), vasculitis (1) or leukaemia (1). All patients had symptoms of acute respiratory illness with
20 one or more of the following features: abnormal chest signs, arterial hypoxaemia, or abnormal chest radiograph. The 10 remaining patients were immunocompetent, undergoing bronchoscopy to investigate
25 various respiratory disorders. Routine microbiological and cytological analysis including methenamine silver staining was performed on each lavage and an aliquot reserved for the DNA amplification study, performed as described in Example 1.

30 Results

On the basis of clinical progress, response to treatment with nebulised pentamidine (20 cases) or cotrimoxazole (7 cases) and results of standard
35 investigation including methenamine silver staining,

the 47 patients were eventually categorised into four groups (Table 4); 16 immunosuppressed patients with a positive diagnosis of pneumocystis pneumonia by silver staining on lavage and response to treatment, 6 immunosuppressed patients with clinical response to treatment, but negative silver stains on lavage, 15 immunosuppressed patients with neither response to treatment nor positive silver staining on lavage and in whom an alternative diagnosis to account for the respiratory disease was available in 12, and the 10 immunocompetent patients from the routine bronchoscopy list. The results of DNA amplification assayed by the visualisation of a 346 base pair DNA band after a) ethidium bromide staining and b) autoradiography after oligoblotting were compared with these clinical data categorisations (Table 4).

No P.carinii DNA was detectable in the samples from the immunocompetent group. All of the 16 immunosuppressed individuals with P.carinii identified by methenamine silver staining on alveolar lavage had amplified P.carinii DNA visible by both ethidium bromide staining and oligoblotting. Of the 6 individuals judged to have had pneumocystis pneumonia by clinical symptoms and response to treatment but not confirmed by identification of parasites in methenamine silver stained lavage samples, 4 were positive using DNA amplification - both by ethidium bromide staining and oligoblotting - and 2 negative by both methods.

Lesser degree of P.carinii infection were detected in the oligoblots - but not by ethidium bromide staining - of the DNA amplification product in lavage samples from 3/15 of the immunosuppressed subjects without pneumocystis pneumonia. The intensity of the signal was less than that obtained from patients with acute P.carinii infection but significantly greater than a barely visible signal obtained in 4

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other samples from this patient group, and from 3 of 12 preliminary washings of the bronchoscope after routine cleaning and sterilisation.

Example 4

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The method of Example 1 was extended to test induced sputum samples. Fifty one episodes of acute ~~respiratory illness in immunosuppressed HIV-infected~~ individuals were studied. Bronchoscopic alveolar
10 lavage was obtained from each patient and in thirty seven instances induced sputum was also obtained. Samples were examined by routine microbiological and cytological methods, including methenamine silver ~~staining for P. carinii~~; a part of each sample was
15 reserved for DNA amplification. DNA was extracted from 1 ml lavage or sputum by proteinase K digestion (1 mg/ml final concentration of proteinase K, in the presence of 10 mmol EDTA, pH 8.0 and 1% weight/volume sodiumdodecylsulphate, at 50°C for 16 hours and
20 phenol/chloroform extraction. DNA amplification was done with the oligonucleotide primers pAZ102-E and pAZ102-H, with denaturation at 94°C for 90 s, annealing at 55°C for 90 s, and extension at 72°C for 2 min (40 cycles). The amplification products were subjected to
25 electrophoresis in 1.5% agarose gel and the specific P. carinii sequence (346 base pairs) was identified by visualisation with ultraviolet light after ethidium bromide staining or by oligohybridisation, after
Southern transfer and autoradiography with the internal
30 primer pAZ102-L2. Scoring of the DNA bands was done without knowledge of the results of silver staining or of final clinical diagnosis, which was assessed by clinical features and response to treatment with co-trimoxazole or pentamidine.

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The results from the paired lavage and sputum

samples are summarised in the table 5 below. All 14 patients who had a final clinical diagnosis of pneumocystis pneumonia and who also had a positive silver stain on lavage, had a strong signal of amplified pneumocystis DNA from both the lavage sample and sputum. In 5 other patients with a final clinical diagnosis of pneumocystis pneumonia but negative silver stains, 4 were strongly positive by DNA amplification in alveolar lavage; 3 of these 4 were also positive in induced sputum. Silver stain was positive in only one third of sputum samples from cases of pneumocystis pneumonia.

Table 5

Final clinical diagnosis (numbers)	DNA amplification positive		silver stain positive	
	sputum	lavage	sputum	lavage
Pneumocystis pneumonia (20)	18	19	7	14
Other diagnoses (17)	1	1	0	0

Positive signals of amplified DNA can be categorised as strong (visible after ethidium bromide staining of the agarose gel) or weak (visible only on autoradiography after oligoblotting). Independent calibration experiments have shown that a strong signal points to 100 organisms or more in a sample, whereas a weak signal indicates from 1-2 organisms up to 100 organisms per sample. In broad terms, it may be said that patients providing samples with strong signals show clinical symptoms of pneumocystis pneumonia,

whereas patients providing samples with weak signals are in the pre-clinical stage. Thus, in 20 cases judged to have clinical pneumocystis pneumonia, a strong DNA amplification signal was obtained in 19 (95%) of the lavage samples and in 18 (90%) of the paired sputum samples. By contrast, microscopy after silver staining could only diagnose 35% of these cases on induced sputum. The sensitivity of the DNA method is therefore excellent; it is unlikely that the single case, negative by both DNA amplification and silver staining on lavage, had pneumocystis pneumonia.

The specificity of DNA amplification may be judged from the results of another study involving 44 patients, in whom the final clinical diagnosis was of another respiratory illness (i.e. not pneumocystis pneumonia). A strong amplification signal was obtained both in the lavage and sputum samples in only one of these 44 patients; this patient had had a previous episode of pneumocystis pneumonia and returned with a further documented episode within ten weeks of the current study.

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30

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Table 1

Comparative sequence of oligonucleotides pAZ102-L1 (rat P. carinii) and pAZ102-L2 (human P. carinii).

pAZ102-L1	5' -	A	T	A	A	G	G	T	G	A	G	G	A	G	T	C	G	A	G	A	G	- 3'
pAZ102-L2	5' -	A	T	A	A	G	G	T	A	G	A	T	A	G	T	C	G	A	A	A	G	- 3'

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Table 2PAZ 112 - Oligos used in PCR

	<u>Name</u>	<u>Sequence</u>	<u>Length</u> mer
5	1F	A G A A C T G G A T T C T T A G A	17
	1R	A G A A G T A T C A A G T T G A T	17
10	9F	C T C A T G C T T C A A G T G A C	17
	3F	C A C T T T T A C T A A T A G C C	17
15	3R	T T G A T T A T C C A A C C A A G A	18
	4F	T A A A T C C A C A T T C A A A G	17
	4R	T G T T T T T A G T T A A C C C T	17
20	5F	T A C G G G A T T G A G A T A A T	17
	5R	T T T A T G A T G G A G T A C C A	17
25	6F	T A T T T G G A A T T G G A T G A	17
	6R	T C T T T G C C T T G T T A G G A	17
	10F	T A G A C G G T C A C A G A G A T C A G	20
30	10R	G A A C G A T T A C T A G C A A T T C C	20

Table 3PCR Results for pAZ 112

	<u>Oligos</u>	<u>Approx. Size of Amplification Product, bp</u>
5	1F + 9F	285
	1F + 1R	610
10	3F + 3R	516
	4F + 4R	198
	5F + 5R	183
15	6F + 6R	148
	10F + 10R	700
20		
25		
30		
35		

Table 4

Results of DNA amplification of *P. carinii* DNA in bronchoalveolar lavage samples; comparison with methenamine silver staining and clinical response to treatment

Patient group (Number of Patients)	Silver Stain	Response to treatment	Positive DNA amplification for <i>P. carinii</i>		Negative DNA amplification for <i>P. carinii</i>
			ethidium bromide stain + Oligoblot	Oligoblot alone	
Immunosuppressed (16) (6) (15)	+	+	16	0	0
	-	+	4	0	2
	-	-	0	3 †	12 ‡
Immunocompetent (10)	-	-	0	0	10

† Alternative diagnosis: *Toxoplasma gondii*; *Mycobacterium avium* intracellular; pulmonary lymphoma

‡ Alternative diagnosis: Endobronchial Kaposi's sarcoma (2), *Mycobacterium avium* intracellular (2), *Mycobacterium avium* intracellular and cytomegalovirus (1), *Salmonella typhimurium* (1), *Pseudomonas putida* (1), *Streptococcus pneumoniae* and *Haemophilus influenzae* (1), bronchiectasis (1), no diagnosis (3)

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CLAIMS

- 5 1. The nucleic acid sequences shown in Figure 1, single and double chain fragments thereof at least 15 nucleotides in length, and nucleic acid sequences and fragments having at least 90% homology thereto.
- 10 2. The nucleic acid sequence shown in Figure 3, single and double chain fragments thereof at least 15 nucleotides in length, and nucleic acid sequences and fragments having at least 90% homology thereto.
3. Peptide sequences transcribed from the nucleic acid sequences claimed in Claim 1 or Claim 2.
- 15 4. A method of assaying a sample of DNA from respiratory secretion of a patient possibly infected with Pneumocystis carinii, which method comprises using a pair of oligonucleotide primers based on the sequences shown in Figure 1 or Figure 3 to amplify by a
- 20 polymerase chain reaction a polynucleotide sequence derived from P. carinii if such sequence is present in the sample, and detecting the amplified sequence if present.
5. A method as claimed in Claim 4, wherein the
- 25 amplified sequence is detected by electrophoresis and staining.
6. A method as claimed in Claim 4, wherein the amplified sequence is detected by hybridisation to a labelled probe which probe is a nucleic acid sequence
- 30 according to Claim 1 or Claim 2.
7. A method as claimed in Claim 7, wherein the probe is the 20-mer.
- 5'- A T A A G G T A G A T A G T C G A A A G - 3'

8. A method as claimed in any one of Claims 4 to 7, wherein the oligonucleotide primers are:-

5' - G A T G G C T G T T T C C A A G C C C A - 3'

5' - G T G T A C G T T G C A A A G T A C T C - 3'.

9. A method as claimed in any one of claims 4 to 8, wherein the respiratory secretion assayed is induced sputum.

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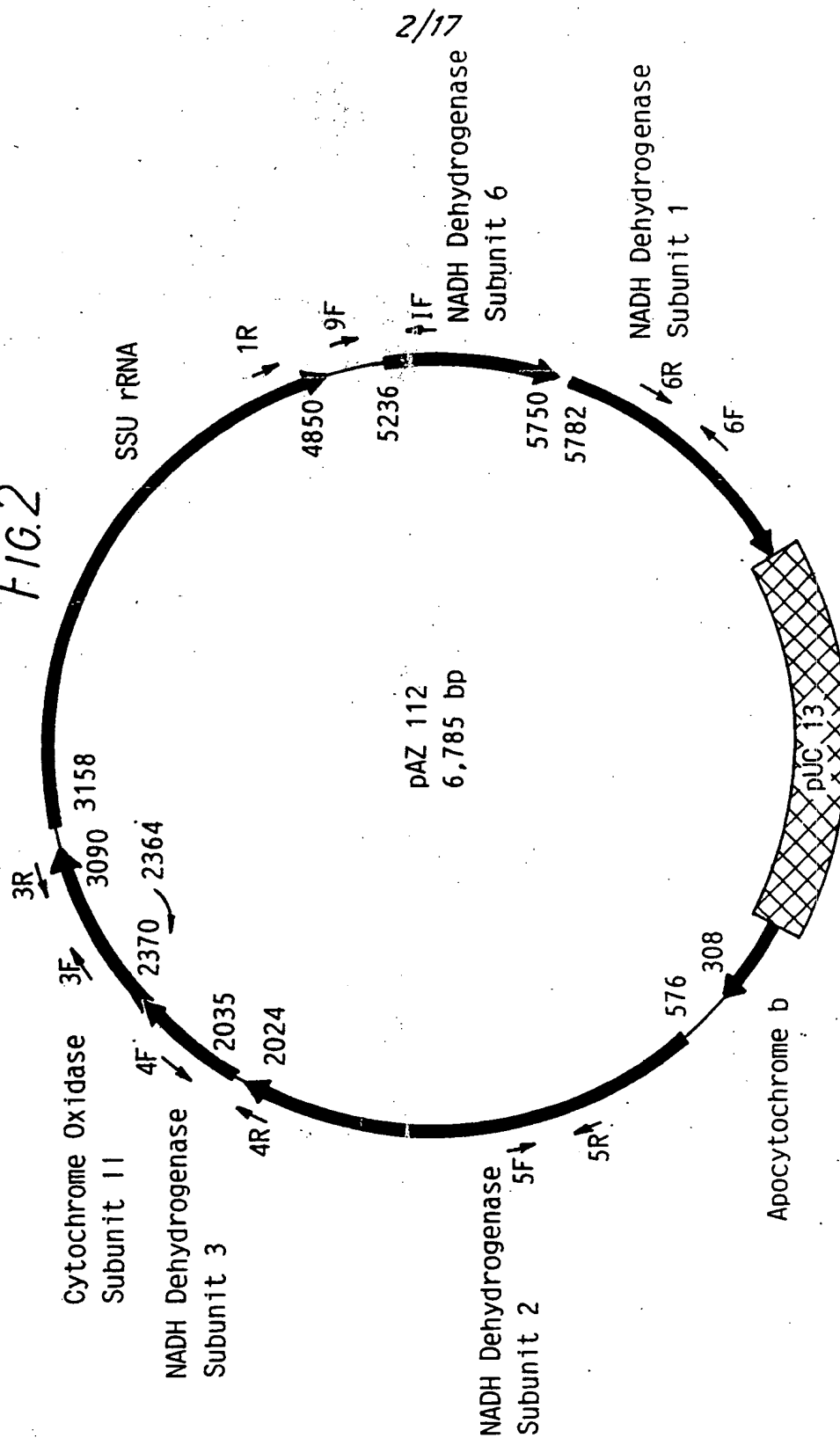
FIG. 1

Rat
Human

TTGIGGTAAGGAGTGAATAACAAATCGGACTAGGATATAGCTGGTTTTCTGCGAAATCT
TTGIGGTAAGGAGTGAATAACAAATCGGACTAGGATATAGCTGGTTTTCTGCGAAATCT
TATTTTGGTATGACCTGCTATTTT--ATTGTGAGTGG--GTTATAGGAGG--TGAATAATCTA
TCTTTTGGCAAA--TTGTTTATTTCTCTCICAA-A-AATAGTAGGTAT---AGGAGTGAATATCTC
ACTTATGTTAGAGGGGAGTATGAAGGTATCTTACTTTGGATATTTAATCTCAGAAATAGCTATTT--
-----GAGGGAGTATGAATAATTTTATCTC-A-GATATTTAACTCAGAAATAGCTATTTCTC
AATATATGATGAGTTATTCAGACTTCTTTCGGATAAGGTGAGGAGTCTGAGGGGAAACAGCCCCAGAA
TTAA--AATGAATAA--TCAGACTTATGTCGATTAAGGTAGATAGTCTGAGAGGGGAAACAGCCCCAGAA
TAAATATAAAGTTCCAAAAATTGTTATTGAGTGAATTTAAAGAAAGTTTCTTCTGTTAGACAGTGA
CAGTAATTAAGCTCCCAATTAATATTTAAGTGAATTAAGAACTTGGTATTTCTTAAGACAGTTGA

AGAAG
AGAAG

FIG. 2



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FIG.3

D L F L I N C * G L * Q * * P Q Y * Y C
 I Y F * * I V R G C S N D S L / N I S I V
S I S D K L L G V V A M I A S I L V L F
 GATCTATTTCTGATAAATTGTTAGGGGTTGTAGCAATGATAGCCTCAATATTAGTATTGT
 10 20 30 40 50 60

S Y Y L S * I Y L E F E D L S F D L * V
 L I T S L R F I * N S R I C L S T S K *
L L P L L D L S R I R G S V F R P L S K
 TCTTATTACCTCTCTTAGATTATCTAGAATTGAGGATCTGTCTTTGACCTCTAAGTA
 70 80 90 100 110 120

N S S F G S L L Q I S Y Y * C T * D L N
 I L L L D L C Y K F L I I N V L R I S T
F F F W I F V T N F L L L M Y L G S Q H
 AATTCTTCTTTTGGATCTTTGTTACAAATTTCTTATTATTAATGTACTTAGGATCTCAAC
 130 140 150 160 170 180

M * K N L I L L L V D M E P Y F T S H I
 C R R T L Y Y Y W * I W N P T L L L I F
V E E P Y I T I G R Y G T L L Y F S Y F
 ATGTAGAAGAACCTTATATTACTATTGGTAGATATGGAACCTACTTTACTTCTCATATT
 190 200 210 220 230 240

L S L * Y Q L * Q * L R I L * Q I * L *
 C L Y S T N Y S S D * E Y F S R S S F N
V F I V P I I A V I E N T L A D L A L T
 TTGTCITTATAGTACCAATTATAGCAGTGATTGAGAATACTTTAGCAGATCTAGCTTTAA
 250 260 270 280 290 300

Q N N S G F L L F K K V F F L E S P I R
 K I I Q D F Y Y L K R Y S F W K V R Y E
K * F R I F I I * K G I L F G K S D T S
 CAAATAATTGAGGATTTTATTATTTAAAAAGGTATTCTTTTGGAAAGTCCGATACGA
 310 320 330 340 350 360

Apocytochrome b

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V K D N R I K S I L * K * K T F R R N *
 L K I I E * N L F C K N R R H L E E T K
 * R * * N K I Y S V K I E D I * K K L K
 GTTAAAGATAATAGAATAAAATCTATTCTGTAAAAATAGAAGACATTTAGAAGAACTAA
 370 380 390 400 410 420

N L Y L * M E L S E P I N L I F V I Y *
 I S I F R W S * A S L L I L S S L F T K
 S L S L D G V K R A Y * S Y L R Y L L N
 AATCTCTATCTTTAGATGGAGTTAAGCGAGCCTATTAATCTTATCTTCGTTATTTACTAA
 430 440 450 460 470 480

T K * Y L N C L V F K L I S I D N * I I
 L S N I * I A W Y S S * F Q * I I K * L
 * V I F K L L G I Q V N F N R * L N N *
 ACTAAGTAATATTTAAATTGCTTGGTATTCAAGTTAATTTCAATAGATAATTAATAATT
 490 500 510 520 530 540

N K Q N S A Y * N L K K C Y Y Q V * L V
 I N R I R L I K I * K N V I I K Y N * S
 * T E F G L L K S E K M L L S S I I S C
 AATAACAGAATTTCGGCTTATTAAATCTGAAAAATGTTATTATCAAGTATAATTAGTC
 550 560 570 580 590 600

S * L L * L F L L L T G I * F C * V E *
 A N C Y S S F F S L E F S F V E * N K
 L I A I A L S S S H W N L V L L S R I S
 AGCTAATTGCTATAGCTCTTTCTTCTCACTGGAATTTAGTTTTGTTGAGTAGAATAA
 610 620 630 640 650 660

V L F P * S I L * F * H I M F I M * R L
 Y Y F L N L F Y N F D I * C L L C R D Y
 I I S L I Y S I I L T Y N V Y V E I I
 GTATTATTTCTTAATCTATTCTATAATTTTGACATATAATGTTTATTATGTAGAGATTA
 670 680 690 700 710 720

* G * V * E S I M D F Y K * L V * H N L
 R V R F R N L * W I F T S N * F N T I C
 G L G L G I Y N G F L Q V T S L T Q F V
 TAGGGTTAGGTTTAGGAATCTATAATGGATTTTACAAGTAACTAGTTTAACACAATTTG
 730 740 750 760 770 780

oligo 8F

L I S L F F S * E F * Y W V L Q G S I M
 * Y L Y F S L R N F N I G Y Y R V L S C
 D I F I F L L G I L I L G I T G F Y H V
 TTGATATCTTTATTTTCTCTTAGGAATTTAATATTGGGTATTACAGGGTTCTATCATG
 790 800 810 820 830 840

NADH dehydrogenase subunit 2

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L T K T I H E N L Q V F M I L N N I * S
 * Q R Q F T R T Y K S L * F * T T F R V
D K D N S R E L T S L Y D F K Q H L E Y
 TTGACAAAGACAATTACAGAGAACTTACAAGTCTTTATGATTTTAAACAACATTAGAGT
 850 860 870 880 890 900

I Q F * V F S Y Y L E V K F * * V H * M
 S S S K S F L I I W K S N S S K F I E C
P V L S L F L L F G S Q I L V S S L N V
 ATCCAGTTCTAAGTCTTTTCTTATTATTGGAAGTCAAATTCTAGTAAGTTCATTGAATG
 910 920 930 940 950 960

* L L S I Y L * N Y K V S L F I S C H L
 D Y F L F I F R I T K F L S L Y L V I F
I T F Y L S L E L Q S F S L Y I L S S L
 TGATTACTTTCTATTTATCTTTAGAATTACAAAGTTTCTCTTTTATATCTTGTCTCTT
 970 980 990 1000 1010 1020

* D P L N K V * N I F Y * G L Y L L A L
 K I L * T R F K I F F I R G S I F L L Y
R S S K Q G L K Y F L L G A L S S C F I
 TAAGATCCTCTAAACAAGGTTTAAATATTTTTATTAGGGGCTCTATCTTCTTGTCTTA
 1030 1040 1050 1060 1070 1080

F Y * D L V W C T V I Q E * H L * N L *
 S I R I W F G V Q L Y R N N I F R I S S
L L G F G L V Y S Y T G I T S L E S L A
 TTCTATTAGGATTGGTTTGGTGTACAGTTATACAGGAATAACATCTTTAGAATCTCTAG
 1090 1100 1110 1120 1130 1140

R Y L V R L I * I F I C R L V Y * F V Y
 D I * * G * S K Y L Y A D * F I N L C I
I F S K V N L N I Y M Q I S L L I C V L
 CGATATTTAGTAAGGTTAATCTAAATATTTATATGCAGATTAGTTTATTAATTTGTGTAT
 1150 1160 1170 1180 1190 1200

* E F S L K * G * Y L S I N G Q S M F M
 R N S L * N R D S T F P S M G N R C L *
G I L F K I G I V P F H Q W A I D V Y D
 TAGGAATTCTCTTTAAATAGGGATAGTACCTTTCCATCAATGGGCAATCGATGTTATG
 1210 1220 1230 1240 1250 1260

oligo 5R

M E Y Q Q * * R P G * Q L * Q K Y L Y *
 W S T N N N N D L V N N F N K N I F I N
G V P T I I T T W L T T L T K I S L L I
 ATGGAGTACCAACAATAATAACGACCTGGTTAACAACCTTTAACAAAATATCTTTATTAA
 1270 1280 1290 1300 1310 1320

oligo 5R

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NADH dehydrogenase subunit 2

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Y S * W S L F I I I H Q K I G Q Q Y * C
 I L N G V Y L S S F I R K L D N N I N V
 F L M E F I Y H H S S E N W T T I L M L
 TATTCTTAATGGAGTTTATTTATCATTCATTCATCAGAAAATTGGACAACAATATTAATGT
 1330 1340 1350 1360 1370 1380

Y Y Q C Y L * * W G L S W D Y L N P V L
 I I S V I C D S G V S P G I I S I P Y *
 L S V L S V I V G S L L G L S Q S R I K
 TATTATCAGTGTATCTGTGATAGTGGGGTCTCTCCTGGGATTATCTCAATCCCGTATTA
 1390 1400 1410 1420 1430 1440

oligo 5F(R/C)

N D Y * S I A W * V M * D F * C Y P Y Q
 T I I N L * H G K S C R I F N A I L I N
 R L L I Y S M V S H V G F L M L S L S I
 AACGATTATTAATCTATAGCATGGTAAGTCATGTAGGATTTTAAATGCTATCCTTATCAA
 1450 1460 1470 1480 1490 1500

* * Q R N L * K H S Y F I * Y N I V * Q
 N D R E I F R S I L I L F S T I * Y N K
 M T E K S L E A F L F Y L V Q Y S I T N
 TAATGACAGAGAAATCTTTAGAAGCATTCTTATTTTATTTAGTACAATATAGTATAACAA
 1510 1520 1530 1540 1550 1560

I * M S F * F * L L W D I S T K I Q I V
 F K C L F N S D C Y G I F L Q K S R * *
 L N V F L I L I A M G Y F Y K N P D S E
 ATTTAAATGTCTTTTAAATTCTGATTGCTATGGGATATTTCTACAAAATCCAGATAGTG
 1570 1580 1590 1600 1610 1620

K I L Q * F I S I V * E V W * E S S P Y
 R F S N N L Y Q * F K R F G E S P A L I
 D S P I I Y I N S L R G L V P V Q P L L
 AAGATTCTCCAATAATTTATATCAATAGTTTAAAGAGTTTGGTGAGAGTCCAGCCCTTAT
 1630 1640 1650 1660 1670 1680

Y L F V * P S L Y Y L W G E Y R L L * D
 I Y L F S H L F T I S G G N T A F Y R I
 S I C L A I S L L S L G G I P P F I G F
 TATCTATTTGTTTAGCCATCTCTTACTATCTCTGGGGGGAATACCGCCTTTTATAGGAT
 1690 1700 1710 1720 1730 1740

F L V S * I F Y I V R * L K D I Y L Y L
 F W * V K Y S I * Y D N S R I F I Y I Y
 F G K L N I L Y S T I T Q G Y L F I S I
 TTTTGGTAAGTTAAATATTCTATATAGTACGATAACTCAAGGATATTTATTTATATCTA
 1750 1760 1770 1780 1790 1800

NADH dehydrogenase subunit 2

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Y F L S * R V F * V * V I I * K W Y N C
 T S C L S E C F K Y K L L F K S G T I V
 L L V L A S V L S I S Y Y L K V V Q L L
 TACTTCTTGTCTTAGCGAGTGTTTTAAGTATAAGTTATTATTTAAAAGTGGTACAATTGT
 1810 1820 1830 1840 1850 1860

Y L W E S L L * V L E I Y R F L H I * V
 I C G R V F F K F * K Y T D F Y I F K Y
 F V G E S S L S F R N I Q I S T Y L S T
 TATTTGTGGGAGAGTCTTCTTTAAGTTTATAGAAATATACAGATTCTACATATTTAAGTA
 1870 1880 1890 1900 1910 1920

H * S V F * L * * * Q C F * L T L I L Y
 I N R C S N F N D S N V F S * P * F Y I
 L I G V L T L M I A M F L V N P D F I L
 CATTAAATCGGTGTCTAACTTTAATGATAGCAATGTTTTAGTTAACCTTGATTTATAT
 1930 1940 1950 1960 1970 1980
 oligo 4R

Y N * * I * Q F V N I L F Y N * * S M Q
 T I N K Y N N L * I F Y S I T S N P C K
 Q L I N I T I C K Y F I L * L V I H A N
 TACAATTAATAAATATAACAATTTGTAAATATTTTATTCTATACTAGTAATCCATGCAA
 1990 2000 2010 2020 2030 2040

I L V I V T V A I S L S L I I L N V L L
 Y * * * L Q * L F H Y H * * Y * M F Y *
 I S N S Y S S Y F I I N N I K C F I S
 ATATTAGTAATAGTTACAGTAGCTATTTTATTATCATTAAATAATATTAATGTTTTATTA
 2050 2060 2070 2080 2090 2100

A K T S P T L E K V S P F E C G F S S F
 L R P L Q H * R K F L P L N V D L V L F
 * D L S N I R E S F S L * M W I * F F S
 GCTAAGACCTCTCCAACATTAGAGAAAGTTTCTCCCTTTGAATGTGGATTGATTTT
 2110 2120 2130 2140 2150 2160
 oligo 4F(R/C)

H Q T R S P F N I Y Y Y L I G L L F L I
 I K R E V L L T F I I I * * V Y Y F * S
 S N A K S F * H L L L F N R F I I S N L
 CATCAACGCGAAGTCCTTTTAACATTTATTATTATTTAATAGGTTTATTATTTCTAATC
 2170 2180 2190 2200 2210 2220

NADH dehydrogenase subunit 2

NADH dehydrogenase subunit 3

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F D L E I L L I Y P Y A L F T T T Y G F
L I * K F Y * F I P M L Y L Q Q P M D F
* F R N F I N L S L C F I Y N N L W I L
TTTGATTAGAAATTTTATTAATTTATCCCTATGCTTTTATTTACAACAACCTATGGATT
2230 2240 2250 2260 2270 2280

Y I F N I F L I F L T I G F I Y E F G K
I Y L I Y F * S F * Q * V L Y T N L A R
Y I * Y I F N L F N N R F Y I R I W Q G
TATATTTAATATATTTTAACTTTTAAACAATAGGTTTATATACGAATTTGGCAAG
2290 2300 2310 2320 2330 2340

G V L K F K T H E * Y Y T * R C T H S L
E F * N L R P M N N I I H N D A P T P W
S F K I * D P * I I L Y I T M H P L L G
GGAGTTTAAATTTAAGACCCATGAATAATATTATACATAACGATGCACCCACTCCTG
2350 2360 2370 2380 2390 2400

G Y I F P R W S E S R L * W Y S R I T *
G I Y F Q D G A S P V Y D G I V E L H D
V Y I S K M E R V P S M M V * * N Y M T
GGGTATATATTTCCAAGATGGAGCGAGTCCCGTCTATGATGGTATAGTAGAATTACATGA
2410 2420 2430 2440 2450 2460

P S S F L L T N S I S R S F L D S V L Y
Q V L F Y L L I V L V G V S W I L F S T
K F F F T Y * * Y * * E F L G F C S L Q
CCAAGTTCTTTTACTTACTAATAGTATTAGTAGGAGTTTCTTGGATTCTGTTCTCTAC
2470 2480 2490 2500 2510 2520

N F T I Q R F R D R P * I S * S * Y N Y
I L R F R G S G I V H K Y H N H S T T I
F Y D S E V Q G S S I N I I I I V Q L *
AATTTTACGATTTCAGAGGTTTCAGGATCGTCCATAAATATCATAATCATAGTACAACTAT
2530 2540 2550 2560 2570 2580

R I C L D S E S S T F T N S H C F S K F
E F V W T V S P A L L I A I A F P S F
N L F G Q * V Q H F Y * * P L L F Q V S
AGAATTTGTTTGGACAGTGAGTCCAGCACTTTTACTAATAGCCATTGCTTTTCCAAGTTT
2590 2600 2610 2620 2630 2640

Q I I V F N G * S D R S I H N N * S D P
K L L Y L M D E V I D P S I T I K A I G
N Y C I * W M K * S I H P * Q L K R * V
CAAATTATTGTATTTAATGGATGAAGTGATCGATCCATCCATAACAATTAAGCGATAGG
2650 2660 2670 2680 2690 2700

oligo3f

NADH dehydrogenase subunit 3

Cytochrome oxidase subunit 11

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S S M V L V L * I L R L Y R Q R G S I Y
 H Q W Y W S Y E Y S D Y T D K E G Q S I
 I N G I G P M N T P I I Q T K R V N L *
 TCATCAATGGTATTGGTCCTATGAATACTCCGATTATACAGACAAAGAGGGTCAATCTAT
 2710 2720 2730 2740 2750 2760

R I * F L Y V T H R R S * G G S I K T I
 E F D S Y M L P T E D L E E G Q L R Q L
 N L I L I C Y P Q K I L R R V N * D N *
 AGAATTTGATTCTTATATGTTACCCACAGAAGATCTTGAGGAGGGTCAATTAAGACAATT
 2770 2780 2790 2800 2810 2820

R G * * P S L S S S E Y S S S I Y Y Y C
 E V D N R V L V P V N T P L R F I I T A
 R L I T E S * F Q * I L L F D L L L L L
 AGAGGTTGATAACCGAGTCTTAGTTCAGTGAATACTCCTCTTCGATTATTATTACTGC
 2830 2840 2850 2860 2870 2880

Y R C F T * F C G S F F R N Q S G C E S
 T D V L H D F A V P S L G I K V D A S P
 Q M F Y M I L R F L L * E S K W M R V Q
 TACAGATGTTTTACATGATTTTGCAGTTCTTCTTTAGGAATCAAAGTGGATGCGAGTCC
 2890 2900 2910 2920 2930 2940

R S I K S S I N I C T T * R S V L W S M
 G R L N Q V S T Y V Q R E G V Y Y G Q C
 V D * I K Y Q H M Y N V K E C I M V N V
 AGGTCGATTAAATCAAGTATCAACATATGTACAACGTGAAGGAGTGTATTATGGTCAATG
 2950 2960 2970 2980 2990 3000

* * T M W C I T * * Y A D C H R G S L F
 S E L C G V L H S S M P I V I E A V S L
 V N Y V V Y Y I V V C R L S S R Q S L *
 TAGTGAACATATGTGGTGTATTACATAGTAGTATGCCGATTGTCATCGAGGCAGTCTCTTT
 3010 3020 3030 3040 3050 3060

R K I F I L V G * S I I H H S S I Y S P
 E K F L S W L D N Q * S I I L P F T L R
 K N F Y L G W I I N N P S F F H L L S V
 AGAAAAATTTTATCTTGGTTGGATAATCAATAATCCATCATTCTTCCATTTACTCTCCG
 3070 3080 3090 3100 3110 3120

oligo 3R (R/C)

Cytochrome oxidase subunit II

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* A K K I D W N R I I Y F * T D S S * K
 E Q R K * I G I E L S I F K R I V H E S
 S K E N R L E * N Y L F L N G * F M K V
 TGAGCAAAGAAAATAGATTGGAATAGAATTATCTATTTTAAACGGATAGTTTCATGAAAG
 3130 3140 3150 3160 3170 3180

S E F N V S S E * T L S R G I T H A N R
 Q S L M L A Q N E R Y L E A L H M Q I V
 R V * C * L R M N A I * R H Y T C K S S
 TCAGAGTTTAATGTTAGCTCAGAATGAACGCTATCTAGAGGCATTACACATGCAAATCGT
 3190 3200 3210 3220 3230 3240

Q G W F T I P T V Y R * V * I G I Y P L
 R G G L P F L R C T G E Y K * E S T H *
 G V V Y H S Y G V Q V S I N R N L P I N
 CAGGGGTGGTTTACCATTCTACGGTGTACAGGTGAGTATAAATAGGAATCTACCCATTA
 3250 3260 3270 3280 3290 3300

T F * E L V D E P I L G K V V G G T K A
 H S K S * W M S L S W G R * L V G Q K L
 I L R V S G * A Y L G E G S W W D K S L
 ACATTCTAAGAGTTAGTGGATGAGCCTATCTTGGGGAAGGTAGTTGGTGGGACAAAAGCT
 3310 3320 3330 3340 3350 3360

Y Q A R E P * S M F E R T S D H I G S E
 T K P E N P S Q C L K E P L T T L A L K
 P S Q R T L V N V * K N L * P H W L * N
 TACCAAGCCAGAGAACCCTAGTCAATGTTTGAAGAAGCTCTGACCACATTGGCTCTGAA
 3370 3380 3390 3400 3410 3420

T I A K I L Y Q G V Q Q * G I L V N D R
 Q * P R F S T R E S S S E E Y W S M I A
 N S Q D S L P G S P A V R N I G Q * S Q
 ACAATAGCCAAGATTCTCTACCAGGGAGTCCAGCAGTGAGGAATATTGGTCAATGATCGC
 3430 3440 3450 3460 3470 3480

K I E P A I * K N F Y I L N K E R M M T
 R L N Q P S R R I F I F * T K R G * * R
 D * T S H L E E F L Y S K Q R E D D D V
 AAGATTGAACCAGCCATCTAGAAGAATTTTATATTCTAAACAAAGAGAGGATGATGACG
 3490 3500 3510 3520 3530 3540

L S L L Q S R P N L V P A V A V I R V R
 Y L C Y S L D P I S C Q Q S R * Y E * G
 I F V T V S T Q S R A S S R G N T S E A
 TTATCTTTGTTACAGTCTCGACCCAATCTCGTGCCAGCAGTCGCGGTAATACGAGTGAGG
 3550 3560 3570 3580 3590 3600

Small subunit rRNA

SUBSTITUTE SHEET

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Q A L F I I T R S K G * V G G Y E L I N
 K R Y S S L L G L K G E * V V M N L L I
 S V I H H Y * V * R V S R W L * T Y * L
 CAAGCGTTATTTCATCATTACTAGGTCTAAAGGGTGAGTAGGTGGTTATGAACCTTATTAAT
 3610 3620 3630 3640 3650 3660

* L E S N R R I K N F G S R D E I R * Y
 N * S R I E E * R I L G V E M K S D D T
 T R V E S K N K E F W E * R * N P M I P
 TAACTAGAGTCGAATCGAAGAATAAAGAATTTTGGGAGTAGAGATGAAATCCGATGATAC
 3670 3680 3690 3700 3710 3720

P K D C S W R K H Y S N Y R L T L R Y E
 Q R T A H G E S I I L I I D * H * G T K
 K G L L M A K A L F * L S T D T E V R K
 CCAAAGGACTGCTCATGGCGAAAGCATTATTCTAATTATCGACTGACACTGAGGTACGAA
 3730 3740 3750 3760 3770 3780

oligo 7R (R/C)

S I R R R K D * I P L * F M L * T M N A
 A * G G A R I R Y P C S L C C K R * M L
 H K E A Q G L D T L V V Y A V N D E C *
 AGCATAAGGAGGCGCAAGGATTAGATACCCCTTGTAGTTTATGCTGTAAACGATGAATGCT
 3790 3800 3810 3820 3830 3840

R N * N T L F * F L W * R L * A F H L R
 E I R I L Y F S F C G E D F K H S T * E
 K L E Y S I L V S V V K T L S I P P E K
 AGAAATTAGAATACTCTATTTTAGTTTCTGTGGTGAAGACTTTAAGCATTCCACCTGAGA
 3850 3860 3870 3880 3890 3900

S T V A R L K L K T L D G H R D Q Q * S
 V L S Q G * N S K H * T V T E I S S E A
 Y C R K A E T Q N I R R S Q R S A V K H
 AGTACTGTGCAAGGCTGAAACTCAAAACATTAGACGGTCACAGAGATCAGCAGTGAAGC
 3910 3920 3930 3940 3950 3960

M L F N S I T H D K S Y H S L Y N K Y F
 C C L I R * P T T N L T T P C I I N I F
 V V * F D N P R Q I L P L L V * * I F S
 ATGTTGTTTAATTGATAACCCACGACAAATCTTACCACTCCTTGATAATAAATATTTT
 3970 3980 3990 4000 4010 4020

P * G I V L T L R N F N * I I T Y I L M
 L K G L F * L * G T L I K * * H I Y L *
 L R D C F D F E E L * L N N N I Y T Y D
 CCTTAAGGGATTGTTTTGACTTTGAGGAACCTTAATTAATAATAACATATATACCTATG
 4030 4040 4050 4060 4070 4080

Small subunit rRNA

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I S L * T Y L Y I F V I R H L N L Y * *
* V F K L T Y I Y L * Y V I * T Y I N N
K S L N L L I Y I C N T S S K L I L I I
ATAAGTCTTTAACTTACTTATATATATTTGTAATACGTCATCTAACTTATATTAATAA
4090 4100 4110 4120 4130 4140

* F N S Y Y * I D D I I V V N V M D S N
N S I V I I E S M T * L L L M * W I V I
I Q * L L L N R * H N C C * C D G * * *
TAATTC AATAGTTATTATTGAATCGATGACATAATTGTTGTTAATGTGATGGATAGTAAT
4150 4160 4170 4180 4190 4200

N N * I L L W V * V T L V I I T N Y Y H
I I K Y C Y G C E * H * * * L I I T I
* L N I V M G V S N I S N N N * L L P L
AATAATTAATATTGTTATGGGTGTGAGTAACATTAGTAATAATACTAATTATTACCAT
4210 4220 4230 4240 4250 4260

Y K P Q * * * L I K * L N G M N D * A I
I N H S N N N * L N N * M E * M I K Q L
* T T V I I I D * I I K W N E * L S N *
TATAAACACAGTAATAATAATTGATTAAATAATTAATGGAATGAATGATTAAGCAATT
4270 4280 4290 4300 4310 4320

S N S L I F I T G V A W L S L V R V V K
V I H * Y L L Q V L H G C L * F V L * N
* F I N I Y Y R C C M A V F S S C C E M
AGTAATTCATTAATATTATTACAGGTGTTGCATGGCTGTCTTTAGTTCGTGTTGTGAAA
4330 4340 4350 4360 4370 4380

oligo 2R

C * V N S E N E R N P Y L Y L K L Y K E
V R L I P K T N A I L I F I * N Y I K R
L G * F R K R T Q S L S L F K T I * R G
TGTTAGGTTAATTCGAAAACGAACGCAATCCTTATCTTTATTTAAACTATATAAGAG
4390 4400 4410 4420 4430 4440

V S F H K K E * L G * R Q V L M T L M E
Y L S I R R N N * G E D K S S * P L W S
I F P * E G I I R V K T S P H D P Y G V
GTATCITTCATAAGAAGGAATAATTAGGGTGAAGACAAGTCCTCATGACCCTTATGGAG
4450 4460 4470 4480 4490 4500

W A T D V P Q I F L Q R E A K M K V * A
G L Q T C H K Y F Y K G K Q R * K S E L
G Y R R A T N I S T K G S K D E S L S *
TGGGCTACAGACGTGCCACAAATATTTCTACAAAGGGAAGCAAGATGAAAGTCTGAGCT
4510 4520 4530 4540 4550 4560

Small subunit rRNA

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N P Q K K * K Y G * E S G T R F F E E G
 I L K R N K S T D K N L E L D S L K K E
 S S K E I K V R I R I W N S I L * R R N
 AATCCTCAAAAGAAATAAAAGTACGGATAAGAAATCTGGAACTCGATTCTTTGAAGAAGGA
 4570 4580 4590 4600 4610 4620

oligo 2F (R/C)

I A S N R S S S R N G E T N I C D V L T
 L L V I V H H Q G T V K R T S V M Y * L
 C * * S F I I K E R * N E H L * C T N Y
 ATTGCTAGTAATCGTTCATCATCAAGGAACGGTGAAACGAACATCTGTGATGTACTAACT
 4630 4640 4650 4660 4670 4680

T R Q A R K S L R S I K L I E F N F * R
 L V K R E N H * E V S S * L N L I S K E
 S S S A K I I K K Y Q V D * I * F L K S
 ACTCGTCAAGCGCGAAAATCATTAGAAGTATCAAGTTGATGAATTTAATTTCTAAAGA
 4690 4700 4710 4720 4730 4740

oligo IR

V K E F N I C R I K G F Q R L F P R N L
 L K N L T S V E S K D F S V Y F L E I C
 * R I * H L * N Q R I S A S I S * K F V
 GTTAAAGAATTTAACATCTGTAGAATCAAAGGATTTGAGCGTCTATTTCTAGAAATTTG
 4750 4760 4770 4780 4790 4800

C * V E I R * L * G N L * L K D * I T H
 A K S K * G S C R G T C S * K I K * P I
 L S R N K V A V G E P V A E E L N N P *
 TGCTAAGTCGAAATAAGGTAGCTGTAGGGGAACCTGTAGCTGAAAGATTATAACCCAT
 4810 4820 4830 4840 4850 4860

N P T S F L K R R I L I V V P R I G C K
 T P P H F L R E E S * * W S Q G * A V N
 P H L I S * E K N L D S G P K D R L * T
 AACCCACCTCATTCTTAAGAGAAGAATCTTGATAGTGGTCCCAAGGATAGGCTGTAA
 4870 4880 4890 4900 4910 4920

P I S F N T C E G S T P S L L I L R L T
 L * V L I L V R L P L F S Y F D * L
 Y K F * Y L * G F D S L S S H T S I D S
 CCTATAAGTTTTAATACTTGTGAGGGTTCGACTCCCTCTCTTCTCATACTTCGATTGACT
 4930 4940 4950 4960 4970 4980

L S L I L R L I F L I L R L S F S H A S
 S L S Y F D L S F S Y F D C L F L M L Q
 L S H T S T Y L S H T S T V F F S C F K
 CTCTCTCATACTTCGACTTATCTTTCTCATACTTCGACTGTCTTTTCTCATGCTTCA
 4990 5000 5010 5020 5030 5040

oligo 9F

Small subunit rRNA

SUBSTITUTE SHEET

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S D S S H L F D L L Y S G * F D Y L F L
 V T L L I Y S I Y F I P N S L I I Y S Y
 * L F S F I R F T L F P I V * L F I P T
AGTGACTCTTCTCATTATTCGATTTACTTTATTCCCAATAGTTTGATTATTTATTCCTA
 5050 5060 5070 5080 5090 5100

L V Q * L C S T L I N L I N S I L M F *
 S F N D F V L L L * I * S I L F L C F E
 R S M T L F Y S Y K F N Q F Y S Y V L N
 CTCGTTCAATGACTTTGTTCTACTCTTATAAATTAATCAATTCTATTCTTATGTTTGA
 5110 5120 5130 5140 5150 5160

I V T I A * W * S K A L L M L Q Q K S D
 * L L * L S G K A K H C * C F N R S P I
 S Y Y S L V V K Q S T V N A S T E V R F
 ATAGTTACTATAGCTTAGTGGTAAAGCAAGCACTGTTAATGCTTCAACAGAAGTCCGAT
 5170 5180 5190 5200 5210 5220

S S * * R M F S L E N S T S C L S I L L
 L L S N E C L V * K T Q Q V A * A F Y *
 F L V T N V * F R K L N K L L E H S I S
 TCTTCTTAGTAACGAATGTTTAGTTTAGAAAACCAACAAGTTGCTTGAGCATTCTATTA
 5230 5240 5250 5260 5270 5280

A I L V V T S K N P V L S L L Y L I G L
 L Y * W * L R I Q F F L Y Y I * L D Y
 Y I S G N F * E S S S F F I I F D W I I
 GCTATATTAGTGGTAACTTCTAAGAATCCAGTCTCTTCTTTATTATATTTGATTGGATTA
 5290 5300 5310 5320 5330 5340

oligo IF(R/C)

F I D I G V Y L I S L Q L T Y L G L S Y
 L * I * V C I * Y L F S * L T * G Y H I
 Y R Y R C V F D I S S V N L L R V I I Y
 TTTATAGATATAGGTGTGATTTGATATCTCTTCAGTTAACTTACTTAGGGTTATCATAT
 5350 5360 5370 5380 5390 5400

I T V Y V G A I A M L F I F V I M M L N
 * L Y M L E L L Q C C L S L * L * C * I
 N C I C W S Y C N V V Y L C N Y D V K Y
 ATAAGTATATGTTGGAGCTATTGCAATGTTGTTTATCTTTGTAATTATGATGTTAAAT
 5410 5420 5430 5440 5450 5460

I Q V V E S K K K T
 S K L * N L K R R C
 P S C R I * K E D K
 ATCCAAGTTGTAAGATCTAAAAAGAAGACAAA
 5470 5480 5490

I P L G L
 V Y P * V Y
 Y T L R F I
 ATACCCTTAGGTTTA
 5510 5520

NADH dehydrogenase 6

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F L G S S L V F T L Y L S L P R E I I M
 F * D L A * Y L H F I Y L Y Q E K * * W
 F R I * L S I Y T L F I S T K R N N N G
 TTTTAGGATCTAGCTTAGTATTTACACTTTATTTATCTCTACCAAGAGAAATAATAATG
 5530 5540 5550 5560 5570 5580

E K Y S H L F S Y F S W E N R I L N P S
 R N I L I S S H T S L G K I E F * I L Q
 E I F S S L L I L L L G K * N F K S F S
 GAGAAATATTCTCATCTCTTCTCATACTTCTCTTGGGAAATAGAATTTTAAATCCTTCA
 5590 5600 5610 5620 5630 5640

V V E I L G K V L Y T D Y S L W L L L I
 S * K Y * V K Y F I Q T I L S G Y C * *
 R R N T R * S T L Y R L F S L V I V N K
 GTCGTAGAAATACTAGGTAAAGTACTTTATACAGACTATTCTCTCTGGTTATTGTTAATA
 5650 5660 5670 5680 5690 5700

S L I L V L A I V G A I S I A A H K E *
 V * Y W C * L L L E P F L * Q L T K N K
 S N I G A S Y C W S H F Y S S S Q R I S
 AGTCTAATATTGGTGCTAGCTATTGTTGGAGCCATTCTATAGCAGCTCACAAAGAATAA
 5710 5720 5730 5740 5750 5760

V N I I H Q L M L F E V L V L I V S V L
 L I L S I N * C Y L K Y * Y * * Y P Y C
 * Y Y P S I N V I * S I S I N S I R T V
 GTTAATATTATCCATCAATTAATGTTATTTGAAGTATTAGTATTAATAGTATCCGTACTG
 5770 5780 5790 5800 5810 5820

L S V A Y L T L A E R K V M G S M Q R R
 * V L L I * P * Q R E K * W D L C K D V
 K C C L S N L S R E K S D G I Y A K T F
 TTAAGTGTGCTTATCTAACCTTAGCAGAGAGAAAAGTGATGGGATCTATGCAAAGACGT
 5830 5840 5850 5860 5870 5880

L G P N A V G Y Y G L L Q P F A D A L K
 * D R M P * D I M V Y Y N P L Q M P * N
 R T E C R R I L W F I T T L C R C L K I
 TTAGGACCGAATGCCGTAGGATATTATGGTTTATTACAACCCTTTGCAGATGCCTTAAAA
 5890 5900 5910 5920 5930 5940

L I V K E T I I P S Q A N K I L F F L G
 * * * K K Q * Y L L K P I K S Y S F * V
 D S E R N N N T F S S Q * N L I L S R S
 TTGATAGTGAAGAAACAATAATACCTTCTCAAGCCAATAAAATCTTATTCTTTCTAGGT
 5950 5960 5970 5980 5990 6000

NADH dehydrogenase 6

NADH dehydrogenase 1

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P M I A L V F A L L G W G L I P Y G P G
 L * L L * S L P C * D G V * Y L M G L G
 Y D C F S L C L V R M G S N T L W A W G
 CCTATGATTGCTTTAGTCTTTGCCTTGTTAGGATGGGGTCTAATACCTTATGGGCCTGGG
 6010 6020 6030 6040 6050 6060

oligo 6R

A T I C D F E L G V L Y S L A I S S V G
 Q Q S V I L N * E F S I V * L F L L * G
 N N L * F * I R S S L * F S Y F F C R G
 GCAACAATCTGTGATTTTGAATTAGGAGTTCTCTATAGTTTAGCTATTTCTTCTGTAGGG
 6070 6080 6090 6100 6110 6120

V Y G I L I G G W S S N S K Y P L V G S
 F T E S * * G V G H P I P N I L * * V L
 L R N L N R G L V I Q F Q I S F S R F S
 GTTTACGGAATCTTAATAGGGGGTTGGTCAATCCAATCCAATATCCTTTAGTAGGTTCT
 6130 6140 6150 6160 6170 6180

oligo 6F(R/C)

L R S T A Q L I S Y E L V L T S I V F I
 * G V Q L N * L V M N * F L L R L Y S S
 K E Y S S I N * L * T S S Y F D C I H H
 CTAAGGAGTACAGCTCAATTAATTAGTTATGAAGTCTTCTTACTTCGATTGTATTTCATC
 6190 6200 6210 6220 6230 6240

I V F F S G T L N W T Q L V E A Q H S I
 L S S S L E L L I G L N W S K L N I L F
 C L L L W N S * L D S I G R S S T F Y L
 ATTGTCTTCTTCTCTGGAAGTCTTAATTGGAGTCAATTGGTCAAGCTCAACATTCTATT
 6250 6260 6270 6280 6290 6300

W Y C I P L L P L F V M Y F I G A L A E
 G I A Y L F Y H F L S C I S L E L * L K
 V L H T S S T T F C H V F H W S F S * N
 TGGTATTGCATACCTCTTCTACCACTTTTTGTGATGATTTTCATTGGAGCTTTAGCTGAA
 6310 6320 6330 6340 6350 6360

T N R A P F D L P E A E S E L V A G F M
 Q I E L L L I Y P K R S L N * L Q D L *
 K S S S F * F T R S G V * I S C R I Y D
 ACAAATCGAGCTCCTTTTGATTTACCGAAGCGGAGTCTGAATTAGTTGCAGGATTTATG
 6370 6380 6390 6400 6410 6420

T E Y S A A I F V Y Y F L A E Y G N I L
 Q N I P Q L Y S Y I T S * Q N T G I F F
 R I F R S Y I R I L L L S R I R E Y S F
 ACAGAATATTCCGCAGCTATATTCTGATATTACTTCTTAGCAGAATACGGGAATATTCTT
 6430 6440 6450 6460 6470 6480

NADH dehydrogenase 1

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L I S T L S V I F F L G G Y L L P F E G
 * S Q H Y Q * S S S W E V I Y Y L S K V
 N L N I I S D L L L G R L F I T F R R L
 TTAATCTCAACATTATCAGTGATCTTCTTCTTGGGAGGTTATTTATTACCTTTTGAAGGT
 6490 6500 6510 6520 6530 6540

C L Q L V G I G L Q S I T G Y R V P I L
 V Y N L L E L V Y S L L L A I E Y L F Y
 S T T C W N W F T V Y Y W L * S T Y F I
 TGTCTACAACTTGTGGAATTGGTTTACAGTCTATTACTGGCTATAGAGTACCTATTTTA
 6550 6560 6570 6580 6590 6600

F L T S S V T E G I F Y G L S L G I K V
 S * L L Q L Q K E S F M V F P * V L K Y
 L N F F S Y R R N L L W S F P R Y * S I
 TTCTTAACCTTCTTCAGTTACAGAAGGAATCTTTATGGTCTTTCCCTAGGTATTAAAGTA
 6610 6620 6630 6640 6650 6660

S L L I F L F I W V R A S F P R I R Y D
 L Y * Y F Y L Y G L E L L S H E * D M I
 F I N I F I Y M G * S F F P T N K I * S
 TCTTTATTAAATATTTTATTTATATGGGTTAGAGCTTCTTTCCACGAATAAGATATGAT
 6670 6680 6690 6700 6710 6720

H I F Q R T L D H K G I I S D I P K D I
 I Y S K G H * I I R A L Y Q I F Q R T L
 Y I P K D I R S * G H Y I R Y S K G H *
 CATATATTCCAAGGACATTAGATCATAAGGGCATTATATCAGATATTCCAAGGACATT
 6730 6740 6750 6760 6770 6780

R
 D
 I
 AGATC

NADH dehydrogenase 1

SUBSTITUTE SHEET

INTERNATIONAL SEARCH REPORT

International Applicat. No. PCT/GB 91/00869

I. CLASSIFICATION	SUBJECT MATTER (if several classification symbols apply, (all)) ⁶		
According to International Patent Classification (IPC) or to both National Classification and -- C			
Int.C1.5	C 12 Q 1/68	C 12 P 19/34	C 07 H 21/04
C 07 K 15/02	// C 12 N 15/11		

II. FIELDS SEARCHED

Minimum Documentation Searched ⁷			
Classification System	Classification Symbols		
Int.C1.5	C 12 Q C 07 K	C 12 P A 61 K	C 12 N
Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched ⁸			


III. DOCUMENTS CONSIDERED TO BE RELEVANT⁹

Category ¹⁰	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³
X	EP,A,0327390 (ISIS INNOVATION LTD) 9 August 1989, see figures 2,3, which correspond to figure 3 of application, nucleotides 1-89,90-539 respectively (cited in the application)	2
Y	---	4,5,6,9
P,X	THE LANCET, vol. 336, no. 8713, August 1990, A.E. WAKEFIELD et al.: "Detection of Pneumocystis carinii with DNA amplification", pages 451-453, see the whole article	2,4-8
P,Y	WO,A,9013669 (XYTRONYX INC.) 15 November 1990, see the whole document -/-	4,5,6,9

- ¹⁰ Special categories of cited documents: 10
- "A" document defining the general state of the art which is not considered to be of particular relevance
 - "E" earlier document but published on or after the international filing date
 - "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
 - "O" document referring to an oral disclosure, use, exhibition or other means
 - "P" document published prior to the international filing date but later than the priority date claimed

- "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step
- "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- "&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
09-09-1991	28.10.91
International Searching Authority	Signature of Authorized Officer
EUROPEAN PATENT OFFICE	 M. van der Drift

III. DOCUMENTS CONSIDERED TO BE RELEVANT

(CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of relevant passages	Relevant to Claim *
A	CHEMICAL ABSTRACTS, vol. 111, no. 3, 3 July 1989, page 205, abstract no. 2066k, (Columbus, Ohio, US), A.E. WAKEFIELD et al.: "Cloning of DNA from <i>Pneumocystis carinii</i> ", & J. PROTOZOOL. 1989, 36(1), 55-75	
P,X	MOLECULAR AND BIOCHEMICAL PARASITOLOGY, vol. 45, no. 1, January 1991, Elsevier Science Publishers B.V. (Biomedical Division), K. SINCLAIR et al.: "Pneumocystis carinii organisms derived from rat and human hosts are genetically distinct", pages 183-184, see the whole article	1-8
P,X	MOLECULAR AND BIOCHEMICAL PARASITOLOGY, vol. 43, no. 1, November 1990, Elsevier Science Publishers B.V. (Biomedical Division), A.E. WAKEFIELD et al.: "Amplification of mitochondrial ribosomal RNA sequences from <i>Pneumocystis carinii</i> DNA rat and human origin", pages 69-76, see the whole article	1-8

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO.

GB 9100869
SA 48160

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 17/10/91. The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A- 0327390	09-08-89	JP-A- 2005899	10-01-90
WO-A- 9013669	15-11-90	AU-A- 5671190	29-11-90

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For more details about this annex : see Official Journal of the European Patent Office, No. 12/82